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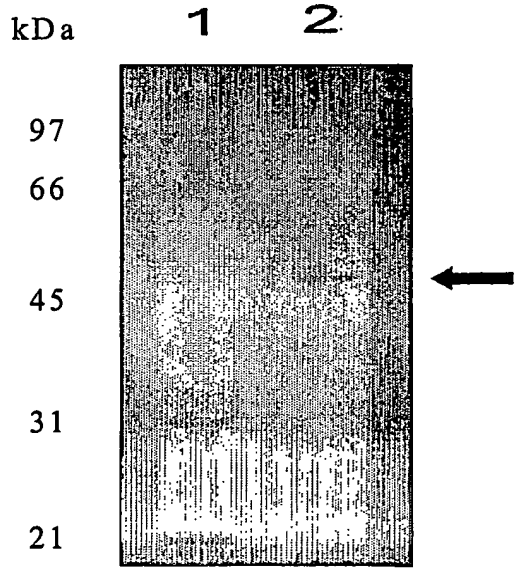
(54) Title: PHYTASE PRODUCED FROM CITROBACTER BRAAKII



(57) Abstract: The present invention relates to novel phytase enzyme, a gene coding the enzyme, and a Citrobacter sp. producing the enzyme. Particularly, the present invention relates to the phytase enzyme produced from Citrobacter sp. having (a) molecular weight of 47 kDa, (b) optimal pH of 3.5-4.5, (c) optimal temperature of 45-55 °C, (d) as substrates phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP, (e) Michaelis constant of 0.3-0.5 mM utilizing phytate as substrate, and (f) high resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin. The present invention also relates to the gene coding the phytase enzyme and the Citrobacter braakii producing the enzyme. The phytase enzyme and the Citrobacter braakii producing the enzyme of the present invention can be used in manufacturing a feed of monogastrics as feed additive and in recovering a specific decomposition product of phytate at low price.

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PHYTASE PRODUCED FROM CITROBACTER BRAAKII

FIELD OF THE INVENTION

The present invention relates to a novel phytase enzyme, a gene coding the enzyme, a *Citrobacter* sp. strain producing the enzyme and a feed additive containing the protein or the strain as an effective ingredient.

BACKGROUND

Phytase is an enzyme decomposing phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate) to produce phosphate and phosphate inositol. Phytic acid takes 50~70% of phosphorus contained in animal feed grains. However, monogastric animals such as fish, fowls and pigs do not have phytase decomposing phytic acid inside body, so that a coefficient of utilization of vegetable phosphorus, which is necessary for growth, is very low, requiring an enough supply from outside body in the form of inorganic compounds. Phytic acid included in feed grains, which is not digested in monogastric animals, can be decomposed enzymatically by microorganisms in

soil or in water while it is in transit to the river and the lake. So, the mass-inflow of phosphorus into underwater environment, where only restricted phosphorus is allowed, causes eutrophication inducing a lack of oxygen and a growth of seaweeds. Phytic acid becomes useless after chelating with important trace minerals, amino acids, vitamins, etc, which means it cannot be used *in vivo* after then, making it an anti-nutrition factor causing a huge nutrition loss in a feed. Thus, if phytase is added to a feed grains for monogastric animals, the useless phytic acid now can be useful, resulting in 1) beneficial reduction of inorganic phosphorus supply, 2) increase of coefficient of utilization of trace bioactive materials, and 3) reduction of phosphorus in animal feces, by which environmental pollution can be reduced. Therefore, the addition of phytase is not only important in economic aspects but also meaningful in environmental protection. Benefits including economic effect of adding phytase are very helpful for preparing globalization.

European countries have been leading the

studies on phytase, so far (A. H. J. Ullah, et al., Biochem. Biophys. Res. Commun. 1999, 264, 201-206; K. C. Ehrich, et al., Biochem. Biophys. Res. Commun.1994, 204(1), 63-68; C. S. Piddington, et al., Gene, 1993, 133(1), 55-62). In particular, they have studied on the effect and functions of phytase extracted from fungi (*Aspergillus* sp.) in monogastric domestic animals and fish (L. G. Young, et al., J Anim Sci 1993, 71(8), 2147-2150; K. D. Roberson, et al., Poult Sci 1994, 73, 1312-1326; N. Simoes, et al., Reprod Nutr Dev, 1998, 38, 429-440; M. Rodehutscord, et al., Arch Tierernahr 1995, 48, 211-219). However, they had troubles in those studies, for example, the amount of phosphorus digested by phytase was limited, the production of phytase was not economical since it was produced mainly in fungi having a long growth term, and the manipulation was troublesome.

Thus, in order to produce a novel phytase having as excellent activity as or different characteristics from the conventional phytase, the present inventors isolated a novel microorganim producing phytase from thousands of strains gathered from seawater and wastewater treatment

plants all over the country and identified thereof. The present inventors completed this invention by confirming that phytase produced by the above microorganism of the invention was a novel protein having a novel base sequence and an excellent titer.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a novel protein decomposing phytic acid produced from a *Citrobacter* sp. strain and a gene coding the protein.

It is also an object of this invention to provide a *Citrobacter braakii* strain producing the above protein.

It is a further object of this invention to provide a feed additive containing the above protein or the above strain as an effective ingredient.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In order to achieve the above object, the present invention provides a protein produced from

a *Citrobacter* sp. Strain and having physicochemical characteristics as follows.

(a) Molecular weight : about 47 kDa on SDS-PAGE,

(b) Optimal pH : pH 3.5 - pH 4.5,

(c) Optimal temperature : 45℃ - 55℃,

(d) Substrate specificity : phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP,

(e) Michaelis constant of 0.3 - 0.5 mM utilizing phytate as a substrate,

(f) High resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin.

The present invention also provides a gene coding the above protein.

The present invention also provides a *Citrobacter braakii* strain producing the above protein.

The present invention further provides a feed additive containing the above protein or the above strain as an effective ingredient.

Hereinafter, the present invention is described in detail.

The present invention provides a novel

protein decomposing phytic acid produced from a *Citrobacter* sp. strain.

The protein having an activity of decomposing phytic acid was named "phytase".

The phytase of the present invention is characterized by having the physicochemical characteristics as follows.

(a) Molecular weight : about 47 kDa on SDS-PAGE,

(b) Optimal pH : pH 3.5 - pH 4.5,

(c) Optimal temperature : 45℃ - 55℃,

(d) Substrate specificity : phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP,

(e) Michaelis constant of 0.3 - 0.5 mM utilizing phytate as a substrate,

(f) High resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin.

Phytase of the present invention is an enzyme having phytase activity, which is originated from *Citrobacter* sp. strain and can be separated and purified after culturing the strain by using ammonium sulfate precipitation, phenyl separose, DEAE-separose, CM-separose and Mono S HR 5/5

column.

The phytase has a molecular weight of 47 kDa on SDS-PAGE and is activated by using phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP as a substrate. The phytase is an acidic enzyme showing a high enzyme activity at 45℃-55℃ (optimal activity is observed at 50℃). The enzyme activity is very stable between pH 3.0 and pH 7.0, the best activity can be seen between pH 3.5 and pH 4.5, and the optimal pH is 4.0. The enzyme activity is strongly inhibited by Fe^{3+}, Zn^{2+} and Cu^{2+} of various metal ions. Km value to phytate is 0.46 mM, and Vmax value is 6,027 U/mg. Besides, the phytase shows a strong resistance against many proteases such as pepsin, trypsin, papain, elastase or pancreatin (see FIG. 4, Table 5 and Table 6).

The phytase of the present invention is produced from *Citrobacter* sp. strain, and is preferably produced from *Citrobacter braakii*. More particularly, it is more preferable for the phytase of the present invention to be produced from *Citrobacter braakii* YH-15 (Accession No: KCCM 10427).

The phytase has an amino acid sequence represented by SEQ. ID. No 2 or a N-terminal amino acid sequence containing a sequence represented by SEQ. ID. No 2 in which one or more amino acids are replaced, deleted or added. The amino acid sequence is quite different from that of conventional phytase enzyme, so that it has been confirmed that the phytase of the present invention is a novel enzyme.

It is more preferable for the phytase of the present invention to include not only a N-terminal amino acid sequence represented by SEQ. ID. No 2 but also an amino acid sequence represented by SEQ. ID. No 7 or to have at least 70% homology with the sequences.

It is also preferred for the phytase of the present invention to have more than 1,500 U/mg of specific activity to phytate and is more preferred to have over 3,000 U/mg of specific activity.

The present invention also provides a gene coding the above protein.

It is preferable for the gene to code an amino acid sequence represented by SEQ. ID. No 7

or at least to code an amino acid sequence having more than 70% sequence homology with the above sequence. It is more preferable for the gene to have a base sequence represented by SEQ. ID. No 6 or to have a base sequence having more than 70% sequence homology with the above.

The phytase of the present invention has an open reading frame for a phytase composed of 1302 bases, and the open reading frame is composed of a signal sequence consisting of 22 amino acids and an active phytase represented by SEQ. ID. No 7 and consisting of 411 amino acids. The molecular weight of an active protein without a signal sequence is about 47,000 Da.

Base sequence of the phytase of the present invention is available for the production of a recombinant protein. For example, the base sequence can be included in various expression vectors for producing an enzyme. And those expression vectors include SV 40 inducer, bacterial plasmid, phage gene, *Baculovirus*, yeast plasmid, recombinant vector constructed by combining a plasmid with phage gene, viral gene,

chromosome, non-chromosome and a synthesized base sequence.

Appropriate host cells can be transfected with the expression vectors to produce a target protein.

Escherichia, Serratia, Corynebacterium, Brevibacterium, Pseudomonas, Bacillus, Aspergillus, Rhizopus, Trichoderma, Neurospora, Mucor, Penicillium, Chluiveromyces, Saccharomyces, Schizosaccharomyces, Pichia sp. are good for host cells.

The present invention further provides *Citrobacter braakii* producing the protein.

Citrobacter braakii YH-15 (Accession No: KCCM 10427) is preferably chosen for *Citrobacter braakii* producing the phytase of the present invention.

The present inventors separated strains, which can produce a phytase decomposing phytate, from a sample taken from seawater and wastewater treatment plants near Busan, Korea. Activities of phytase produced in the strains were measured. And a strain showing the highest phytase activity

was identified by using 16S rRNA sequence analysis and API kit. As a result, the strain of the present invention was confirmed to be a novel strain having 16S rRNA consisting of a base sequence represented by SEQ. ID. No 1, which had 99.0% homology with that of *Citrobacter braakii* and 98% homology with those of *Citrobacter freundii*, *Citrobacter werkmanii* and *Enterobacter aerogenes*.

The strain was a Gram-negative, rod-type bacterium having a cell size of 0.5~1.4 ㎛ and had a flagellum (see FIG. 1). From the investigation of biochemical and physiological characteristics of the strain, the strain was confirmed to be a facultative microorganism, meaning that it could be growing with or without air, was positive to ornithin decarboxylase, and had an ability of citrate utilization but was negative to indole generation, acetone generation, hydrogen sulfide generation, gelatin liquefaction and lysine decarboxilase (see Table 2).

Based on the results of 16S rDNA analysis and morphological and physiochemical characteristics of the strain, the present inventors identified the strain separated in the present invention to

be a novel *Citrobacter brakii*, which was then named "*Citrobacter braakii* YH-15" and was deposited at Korean Culture Center of Microorganisms (KCCM), on September 26, 2002 (Accession No: KCCM 10427).

The present invention also provides a feed additive containing the protein produced from *Citrobacter braakii* or from the strain of the present invention.

The feed additive of the present invention preferably contained *Citrobacter braakii* (Accession No: KCCM 10427) or phytase produced from the strain as an effective ingredient. The feed additive of the present invention can be effectively used for the production of animal feeds since it contained phytase enhancing utilization of phosphorus in feeding grains.

The feed additive of the present invention can be prepared in the form of dried or liquid formulation, and can additionally include one or more enzyme preparations. The additional enzyme preparation can also be in the form of dried or liquid formulation and can be selected from a

group consisting of lipolytic enzymes like lipase and glucose-producing enzymes such as amylase hydrolyzing α -1,4-glycoside bond of starch and glycogen, phosphatase hydrolyzing organic phosphate, carboxymethylcellulase decomposing cellulose, xylanase decomposing xylose, maltase hydrolyzing maltose into two glucoses and invertase hydrolyzing saccharose into glucose-fructose mixture.

The feed additive of the present invention can additionally include other non-pathogenic microorganisms, in addition to phytase or a microorganim producing phytase. The additional microorganism can be selected from a group consisting of *Bacillus subtilis* that can produce protease, lipase and invertase, *Lactobacillus* sp. strain having an ability to decompose organic compounds and physiological activity under anaerobic conditions, filamentous fungi like *Aspergillus oryzae* (Slyter, L. L., *J. Animal Sci.* 1976, 43. 910-926) that increases the weight of domestic animals, enhances milk production and helps digestion and absorptiveness of feeds, and yeast like *Saccharomyces cerevisiae* (Jhonson, D. E., et al., *J. Anim. Sci.*, 1983, 56, 735-739 ;

Williams, P. E. V., et al., 1990, 211).

BRIEF DESCRIPTION OF THE DRAWINGS

The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

FIG. 1 is an electron microphotograph showing the *Citrobacter braakii* cell,

FIG. 2 is a graph showing the cell growth and the enzyme activity of phytase produced from *Citrobacter braakii* YH-15,

FIG. 3 is an electrophoresis photograph showing the result of SDS-PAGE with phytase produced from *Citrobacter braakii* YH-15,

Lane 1 : Marker, Lane 2 : Purified phytase

FIG. 4 is a set of graphs showing the biochemical characteristics of phytase produced from *Citrobacter braakii* YH-15,

A: Relative activity according to pH,

B: Relative activity according to temperature

FIG. 5 is a photograph showing the result of Southern hybridization with a probe using base sequence of phytase, performed after DNA of *Citrobacter braakii* YH-15 was purified.

Lane 1: *Eco*RI and *Xho*I treated,

Lane 2: *Eco*RI treated,

Lane 3: *Sph*I treated,

Lane 4: *Bam*HI and *Hind*III treated,

Lane 5: *Eco*RI and *Hind*III treated,

Lane 6: *Eco*R I and *Bam*H I treated,

Lane 7: *Pst*I treated

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Separation of phytase-producing strains

The present inventors separated phytase-producing strains from samples taken from seawater and wastewater treatment plants near Busan, Korea. Particularly, in order to find phytase-producing strains, samples were taken from wastewater treatment plants near entry of Gwanganli beach and seawater near Busan, Korea, for example, Songjung, Haeundae, Daebyun, Sinsundae, Iegidae, Nakdong estuary, etc. The samples were smeared on artificial seawater plate media, followed by cultivation in a 30℃ incubator for 18 hours. Then, different colonies in various forms were selected. Each colony was smeared on PSM medium (1.5% D-glucose, 0.5% calcium phytate, 0.5% NH_4NO_3, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot 4H_2O$) containing 1.5% agar, followed by cultivation at 30℃ for 2 days. Strains having clear zones, which were generated around colonies, were primarily selected. The selected strain was inoculated in 5 ㎖ of artificial seawater and PSM medium, which were cultured in a 30℃ shaking incubator for 24 hours. Phytase activities in the culture solution and in cell precipitate were

measured and 5 out of the selected strains, which showed high phytase activity, were secondly selected. The present inventors named the 5 selected strains as 'YH-11', 'YH-13', 'YH-15', 'YH-60' and 'YH-103' of our own accord.

The present inventors measured the activity of phytase produced by the 5 strains above (Table 1). Inorganic phosphorus quantitative method of Fiske, *et al.* was used for measuring the activity of phytase in culture solution and in cell precipitate. Particularly, 400 ㎕ of substrate solution (2 mM sodium phytate in 0.1 M sodium acetate buffer, pH 5.0) was added to 100 ㎕ of enzyme solution diluted by required dilution ratio, which was reacted at 37℃ for 30 minutes. Then, 500 ㎕ of 5% TCA solution was added thereto, which was just left at 0℃ for 10 minutes to stop the reaction. As for a control (blank), TCA (trichloroacetic acid) solution was added to enzyme solution to inactivate the enzyme and then substrate solution was added thereto, which was left for a while. 4 ㎖ of reagent A (1:1:1:2 ratio of 6 N H_2SO_4/2.5% ammonium molybdate/10% ascorbic acid/H_2O) was added, followed by reaction at 37℃ for 30 minutes. Then, activities in enzyme

solution and in a control were measured at 820 nm. 1 unit of the enzyme was determined to be the enzyme amount releasing 1 μ mole of phosphate for 1 minute.

From measuring the phytase activity, it was confirmed that phytase produced by YH-15 strain had the highest enzyme activity (Table 1).

<Table 1>

Activity of phytase produced by the selected strain

Strain	YH-11	YH-13	YH-15	YH-60	YH-103
Phytase activity	0.048 U/mℓ	0.041 U/mℓ	0.074 U/mℓ	0.052 U/mℓ	0.044 U/mℓ

Example 2: Analysis of characteristics of YH-15 strain producing a phytase

The present inventors analyzed characteristics of YH-15 strain, which was separated in the above Example 1, producing a phytase having the highest enzyme activity.

YH-15 strain was confirmed to be a gram-negative bacterium through Gram staining. The

strain was a rod type bacterium having a flagellum and the cell size was 0.5 ~ 1.4 ㎛, which was observed under an electron microscope (FIG. 1). The present inventors further investigated biochemical and physiological characteristics of the strain. As a result, the strain was a gram-negative, facultatively aerobic microorganism that could be growing with or without oxygens and showed positive reaction to ornithin decarboxilase but was negative to indole generation. Other biochemical and physiological characteristics of the strain were shown in Table 2. The present inventors also analyzed 16S rRNA sequence of the strain, resulting in that the strain had a base sequence represented by SEQ. ID. No 1 and the base sequence of 16S rRNA showed 99% homology with that of *Citrobacter braakii* and 98% homology with sequences of *Citrobacter freundii*, *Citrobacter werkmanii* and *Enterobacter aerogenes*.

Based on the results of investigation on morphological, physiological and biochemical characteristics and 16S rDNA of the strain, the present inventors identified the strain as a novel *Citrobacter braakii*.

The present inventors named the strain "*Citrobacter braakii* YH-15" and deposited it at Korean Culture Center of Microorganisms (KCCM), on September 26, 2002 (Accession No: KCCM 10427).

<Table 2>

Characteristics of *Citrobacter braakii* YH-15

Characteristics	*Citrobacter braakii* YH-15
Gram-staining	Negative
Morphology and size	0.5× 1.4 ㎛
Mobility	+
Citrate utilization	+
Indole generation	-
Acetone generation	-
Hydrogen sulfide generation	-
Gelatin liquefaction	-
Ornithin decarboxilase	+
Lysine decarboxilase	-

Example 3: Separation and purification of phytase produced by *Citrobacter braakii* YH-15

In order to purify the phytase produced by *Citrobacter braakii* YH-15 strain identified in the above Example 2, the present inventors cultured

the strain under the optimal culture conditions and separated the enzyme.

<3-1> Production of phytase

Citrobacter braakii YH-15 of the present invention was cultured in LB medium containing 1% tryptone, 0.5% yeast extract and 0.5% NaCl at 30℃ for 15 hours, which was called seed-culture solution. The seed-culture solution was inoculated again (1%) to produce the enzyme. The phytase activity was measured with the same method as used in the above Example 1. As a result, the highest phytase activity was observed 16 hours later and at that time the produced enzyme was 0.2 unit/mℓ.

<3-2> Separation and purification of phytase

The present inventors purified phytase produced by *Citrobacter braakii* YH-15. Particularly, cells collected by centrifugation after being cultured in the above Example <3-1> were dissolved in 20 mM sodium acetate (pH 5.0) buffer solution, followed by crushing with a cell homogenizer (30 kHz, 30 minutes). Supernatant was

obtained by centrifugation with 12,000 g for 20 minutes. Ammonium sulfate powder was added to the supernatant, leading to 70% saturation, followed by centrifugation with 12,000 for 20 minutes. Then, precipitate was obtained. Sodium acetate buffer solution (pH 5.0) was added to the precipitate to dissolve it. Dialysis was performed by using the same buffer solution. After dialysis, the solution was centrifuged and supernatant was obtained. Finally, phytase was purified through phenyl-, DEAE- and CM-Sepharose column and Mono S HR 5/5 column.

First, purification by using phenyl-sepharose column was as follows. Phenyl-sepharose column was equilibrated with sodium acetate buffer solution (pH 5.0) supplemented with 1.5 M ammonium sulfate. Enzyme extract solution containing the same amount of ammonium sulfate was added thereto. Then, the column was washed enough with the same buffer solution. While the buffer solution was added to the column, the concentration of ammonium sulfate decreased from 0.5 M to 0 M degree by degree in order to elute bound proteins gradually. 0.3 M ammonium sulfate was used to elute phytase.

Second, purification by using DEAE column was as follows. Phytase solution, which was obtained through phenyl-sepharose column, was equilibrated with tris buffer solution (50 mM Tris-HCl, pH 8.0) by dialysis. The phytase solution was added to DEAE-sepharose column that was equilibrated with the same buffer solution. The same buffer solution was continuously added to separate non-binding fractions showing high phytase activity. The fractions were concentrated and 20 mM sodium acetate (pH 5.0) was used for CM-sepharose column. After washing the column enough with the same buffer solution, bound proteins were eluted by increasing the concentration of NaCl from 0 M to 1 M gradually. At that time, 0.6 M of NaCl was used to elute the proteins.

Lastly, chromatography was performed by using Mono S HR 5/5 FPLC column with the same buffer solution that was used in the purification by using CM-sepharose column. At that time, 0.1 M NaCl was used to elute phytase and the separated phytase was finally purified.

<3-3> Measurement of phytase activity

The enzyme activity of phytase included in each sample prepared from each purification stage of the above Example <3-2> was investigated (Table 3). Protein content was quantified by BCA protein quantification kit provided by Sigma, co. At that time, BSA (bovine serum albumin) was used as a standard protein. Specific activity of the purified phytase to phytate was 3,457 units/mg, recovery rate was 28%, and the final phytase was purified by 12,950 fold (FIG.2).

<Table 3>

Total content, activity, purification rate and recovery rate of phytase purified from *Citrobacter braakii* YH-15

Purification stage	Total activity (U)	Total content (mg)	Specific activity (U/mg)	Concentration (fold)	Recovery rate (%)
Cell homogenate	1,453	5,443	0.27	1.00	100
Ammonium sulfate precipitate	1,380	1,593	0.87	3.25	95

Phenyl-sepharose	941	72.19	13.04	48.85	65
DEAE-sepharose	756	17.19	43.98	164	52
CM-sepharose	459	0.71	646	2,421	32
Mono S HR 5/5	413	0.12	3,457	12,950	28

Example 4: Characteristics of phytase

<4-1> Determination of molecular weight and N-terminal amino acid sequence of phytase

The present inventors measured molecular weight of the purified phytase by SDS-PAGE electrophoresis. In FIG. 3, lane 1 was marker protein whose size was known, lane 2 was the final phytase protein purified through chromatography using Mono S column. From the measurement, phytase of the present invention was confirmed to have molecular weight of about 47,000 Da.

N-terminal amino acid sequence of the phytase protein of the present invention was examined by using protein/peptide sequencer (Applied Biosystem, USA), resulting in the confirmation that N-terminal had an amino acid sequence represented by

SEQ. ID. No 2. N-terminal sequence represented by SEQ. ID. No 2 was compared with N-terminal sequences of *Eschelichia coli* originated phytase enzyme (R. Greiner, et al., Arch. Biochem. Biophys. 1993, 303, 107-113), *Aspergillus ficuum* (A.H. Ullah, et al., Prep. Biochem. 1988, 18, 443-458) originated phytase enzyme and *Bacillus* sp. originated phytase enzyme (Y.O. Kim, et al., FEMS Microbiol Lett, 1998, 162, 185-191), resulting in no similarity among them (Table 4). Therefore, phytase produced by *Citrobacter braakii* YH-15 of the present invention was confirmed to be a novel enzyme.

<Table 4>

Comparison of N-terminal amino acid sequences of the novel enzyme and conventional enzymes

Enzyme	N-terminal amino acid sequence
Citrobacter braakii YH-15 originated phytase	SEQ. ID. No 2 (E-E-Q-N-G-M-K-L-E-R)
Eschelichia coli originated phytase	SEQ. ID. No 3 (S-E-P-E-L-K-L-E-N-A-V-V)
Aspergillus ficuum originated phytase	SEQ. ID. No 4 (F-S-Y-G-A-A-I-P-Q-S-T-Q-E-K-Q)
Bacillus sp. originated phytase	SEQ. ID. No 5 (S-D-P-Y-H-F-T-V-N-A-A-X-E-T-E)

<4-2> Enzyme activity of phytase according to temperature and pH

The present invention investigated an enzyme activity of phytase, according to temperature and pH, purified through chromatography using Mono S column.

FIG. 4A shows the enzyme activity varied with temperature. The highest activity was observed at 50℃. The activity was stably maintained at 50℃ for 1 hour. When the enzyme was left at 55℃ for 10 minutes, 75% of the activity was still remained.

FIG. 4B shows the enzyme activity varied with pH. The highest activity was observed at pH 4.0. 50% of the enzyme activity was still maintained at pH 2.5. The activity was very stably maintained at 37℃, at pH 3.0-4.5 for 7 days, and 50% activity still remained at pH 7.0. But, as the protein was left under pH 3.0 for 4 hours, the enzyme activity was almost lost. From temperature and pH test with the protein, phytase of the present invention was believed to be very suitable for being used as a feed additive for monogastric

animals.

<4-3> Enzyme activity of phytase according to metal ions and inhibitors

The present inventors investigated the effect of metal ions and inhibitors on the enzyme activity of phytase of the present invention. Among various metal ions, the enzyme activity of the protein was strongly inhibited by Fe^{3+}, Zn^{2+} and Cu^{2+} under the concentration of 10 mM and was inhibited 50% by NaCl at the concentration of 1 M (Table 5).

As for inhibitors, the enzyme activity was hardly affected by dithothreitol and 2-mercaptoethanol involved in disulfate bond. But, as the protein was left at 37℃ for 2 hours with 8 M urea or 0.0024% SDS, the enzyme activity was almost lost.

<Table 5>

Enzyme activity of YH-15 phytase according to metal ions and inhibitors

Metal ion or inhibitor	Concentration (mM)	Relative activity (%)

-		100
EDTA	6	98
KCl	6	95
$MgCl_2$	6	71
$ZnSO_4$	8	33
$FeCl_3$	6	19
$MnCl_2$	6	92
$CuSO_4$	6	38
$NiSO_4$	6	88
$CaCl_2$	6	87
$CdCl_2$	6	101
NaCl	6	102
	1000	54

<4-4> Substrate specificity of phytase

Substrate specificity of phytase to various phosphate ester compounds was investigated. As shown in Table 6, phytase had a strong ability to decompose phytate specifically, but could hardly decompose other phosphate ester compounds. Km value to sodium phytate was 0.46 mM and Vmax value was 6,027 U/mg.

<Table 6>

Substrate specificity of YH-15 phytase

Substrate	Relative activity (%)

Phytate	100
p-nitrophenyl phosphate	11.27
Tetrasodium pyrophosphate	5.95
ATP	1.86
ADP	1.04
Glycerophosphate	0.57
Glucose-1-phosphate	0.42
Glucose-6-phosphate	0.33
Fructose-6-phosphate	0.75
Mannose-6-phosphate	0.01

<4-5> Effect of proteases on the enzyme activity of phytase

The present inventors investigated the effect of proteases on the enzyme activity of phytase. Particularly, phytase was left at 37℃ for 2 hours with pepsin and trypsin, resulting in no changes in the enzyme activity. But, as papain, elastase and pancreatin were added, 70~85% of the enzyme activity remained.

The result suggested that phytase could promote coefficient of the enzyme inside monogastric animals owing to its resistance against proteases existed in intestines or stomach.

Example 5: Cloning of phytase gene and base sequencing of the same

Oligonucleotide probe was designed on the basis of an amino acid sequence represented by SEQ. ID. No 2 and was synthesized by using a DNA synthesizer (Applied Biosystems ABI 380B DNA synthesizer).

Citrobacter braakii originated chromosomal DNA was separated, which was then digested with restriction enzymes *Eco*RI and *Xho*I, *Eco*RI, *Sph*I, *Bam*HI and *Hind*III, *Eco*RI and *Hind*III, *Eco*R I and *Bam*H I, and *Pst*I. After electrophoresis, the digested DNA fragments were transferred on nylon membrane.

Oligonucleotide represented by SEQ. ID. No 8, synthesized above, was labeled with DIG, followed by Southern hybridization. As a result, signals were observed at 7.5 kb as *Pst* I was used and at 4.5 kb as *Eco*RI and *Bam*HI were used (FIG. 5).

<5-1> Cloning of phytase gene

Citrobacter braakii originated chromosomal DNA was digested with *Pst* I and only 7.5 kb fragments were separated. After being digested

with *Pst* I again, the above DNA was inserted in pBluscript SK vector (STRATAGENE, USA) pre-treated with phosphatase (calf intestinal phosphatase) to transfect *E. coli* XL1-Blue (STRATAGENE, USA). The transfected strains were smeared on 1.5% agar LB plate supplemented with ampicillin, 1% trypton, 0.5% yeast extract and 0.5% NaCl, after which colonies were transferred onto nylon membrane. Colony hybridization was performed by using the oligonucleotide probe to select colonies showing positive reaction, and plasmids were isolated.

As a result, a 10.5 kb size plasmid containing 7.5 kb DNA insert was confirmed and named pB-phyF.

E. coli XL1-Blue was transfected again with the pB-phyF. Then, phytase activity was measured by the same method as used in the above Example <3-3>. As a result, all of the generated colonies showed phytase activities.

<5-2> Sequence analysis of a novel phytase gene

Base sequence of pB-phyF separated in the above Example <5-1> was analyzed. At that time, DNA sequencing kit (Big Dye DNA Sequencing kit,

Perkin-Elmer, Applied Biosystem) and ABI PRISM DNA sequencer (Perkin-Elmer) were used. The base sequence analyzed by the above automatic sequencer was inputted in DNASTAR amino acid sequence analysis program (DNASTAR, Inc.), by which an open reading frame of phytase represented by SEQ. ID. No 6 composing 1302 bases was determined. The open reading frame was composed of a signal sequence consisting of 22 amino acids and an active phytase consisting of 411 amino acids. The molecular weight of the active phytase without a signal sequence was about 47,000 Da.

The amino acid sequence of a novel phytase obtained above was compared with amino acid sequences recorded in GenBank and SWISSPROT using BLAST program. As a result, it was confirmed that the novel phytase sequence had a very low homology (just 60%) with the sequence originated from *Escherichia coli*. Therefore, the phytase of the present invention produced by *Citrobacter braakii* was confirmed to be a novel enzyme.

INDUSTRIAL APPLICABILITY

As explained hereinbefore, *Citrobacter braakii* of the present invention produces a novel phytase having a strong enzyme activity, comparing to other conventional phytases. Thus, the phytase of the present invention or *Citrobacter braakii* producing the same can be effectively used as a feed additive for monogastric animals and for the recovery of specific degradation product of phytic acid at low price. In addition, the phytase of the present invention has strong resistance against proteases, so that it maintains high enzyme activity without being decomposed in intestines or stomach after being administered in monogastric animals.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in

the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Kim Young-Ok
Biotechnology Research Center. National
Fisheries Research and Development Institute.
408-1. Shirang-ri. Gijang-up. Gijang-gun.
Busan 619-902. Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : *Citrobacter braakii* YH-15	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10427

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☐ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Sep. 26. 2002. (date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361-221. Yurim B/D Hongje-1-dong. Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: Oct. 2. 2002

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the International depositary authority.

Form BP/4 Sole page

What is claimed is

1. A protein having the following characteristics

(a) Molecular weight : about 47 kDa on SDS-PAGE,

(b) Optimal pH : pH 3.5 - pH 4.5,

(c) Optimal temperature : 45℃ - 55℃,

(d) Substrate specificity : phytate, p-nitrophenyl phosphate, tetrasodium pyrophosphate, ATP or ADP,

(e) Michaelis constant of 0.3 - 0.5 mM utilizing phytate as a substrate,

(f) High resistance to protease such as pepsin, trypsin, papain, elastase or pancreatin.

2. The protein as set forth in claim 1, wherein the protein contains an amino acid sequence represented by SEQ. ID. No 2 at N-terminal.

3. The protein as set forth in claim 2, wherein the protein contains an amino acid sequence represented by SEQ. ID. No 7 or an amino acid sequence having over 70% sequence homology with the same.

4. The protein as set forth in claim 2 or claim 3, wherein the specific activity of the protein to phytate is over 1,500 units/mg.

5. The protein as set forth in claim 4, wherein the specific activity of the protein to phytate is over 3,000 units/mg.

6. A gene coding the protein of claim 3.

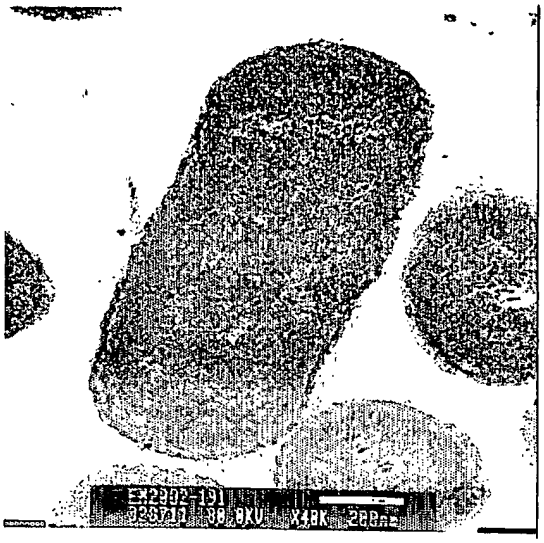
7. The gene as set forth in claim 6, wherein the gene has a base sequence represented by SEQ. ID. No 6 or a base sequence having over 70% sequence homology with the same.

8. A *Citrobacter braakii* YH-15 strain producing the protein of claim 1 (Accession No: KCCM 10427).

9. A feed additive containing the strain of claim 8 or the protein of claim 1 as an effective ingredient.

FIGURES

FIG. 1



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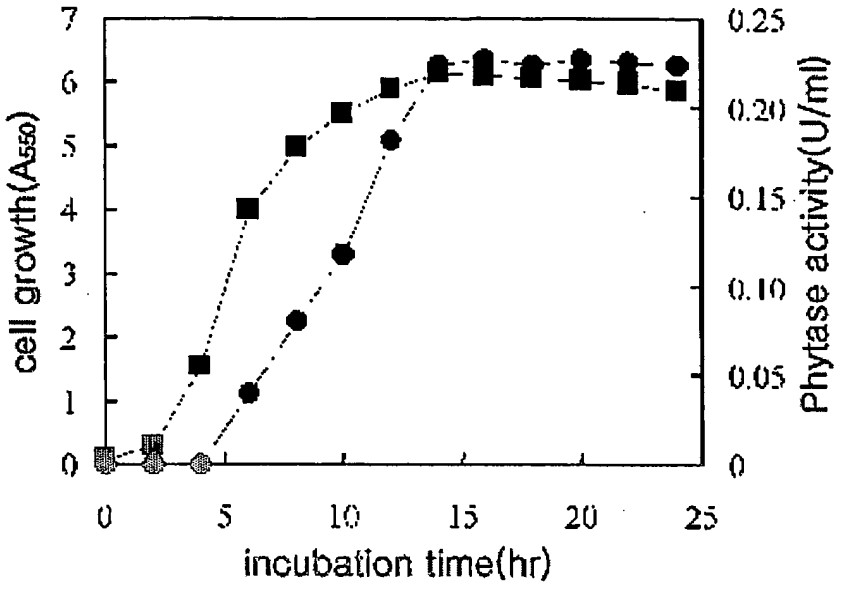
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Phytase activity(U/ml)
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2
3
4
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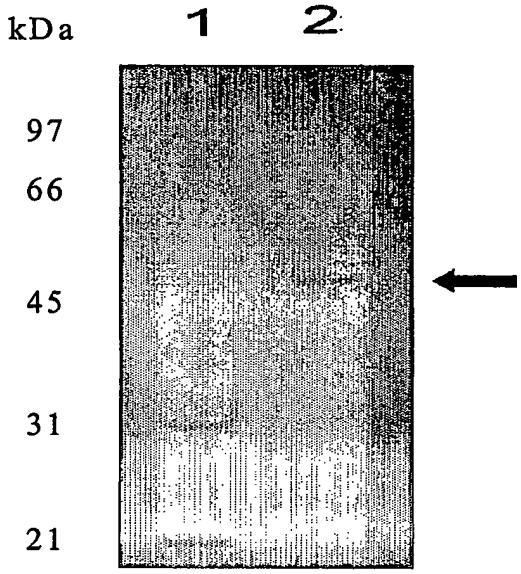
FIG. 3



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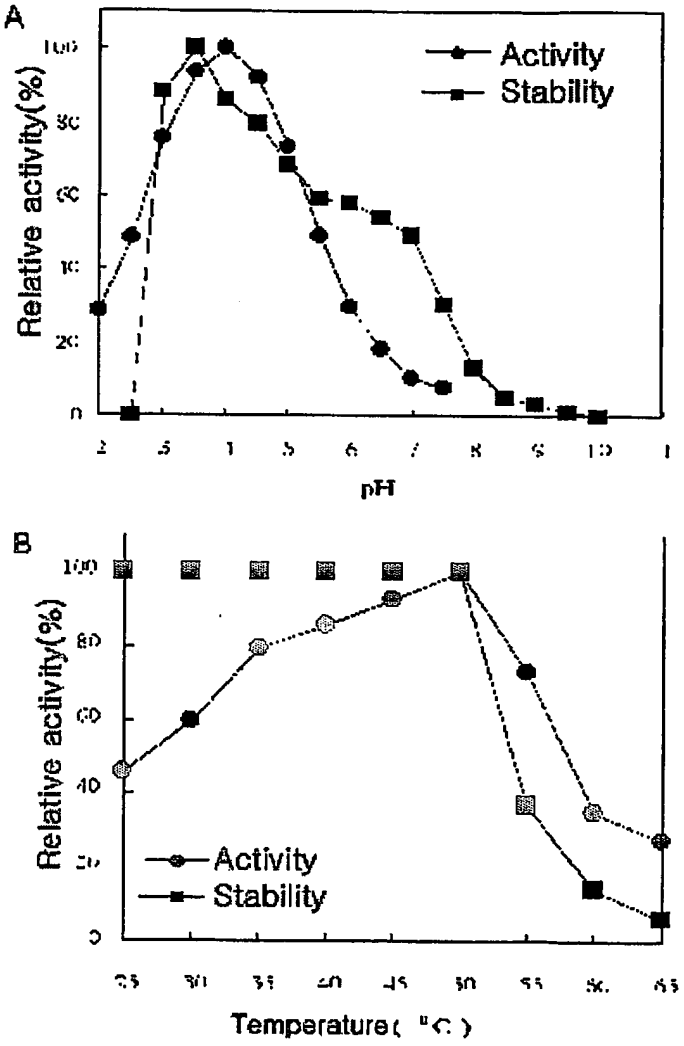
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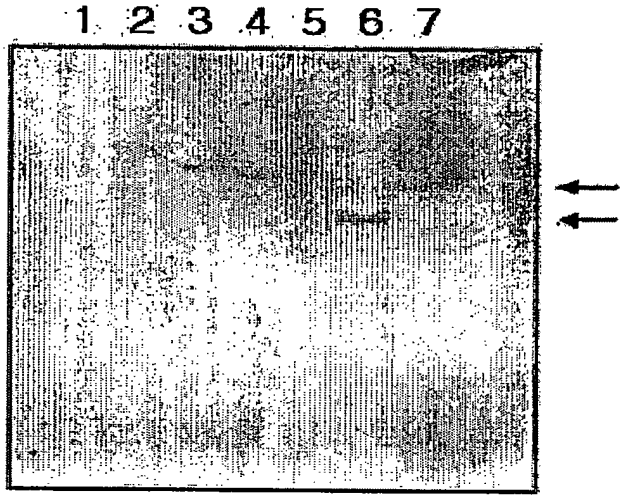
Stability
Relative activity(%)
pH
B
Relative activity(%)
Activity
Stability
Temperature(°C)

FIG. 5



SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2004/000680

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 9/14, C12N 15/52, A23K 1/165

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 9/14, C12N 15/52, A23K 1/165, C12N 1/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the internationl search (name of data base and, where practicable, search terms used)

NCBI PubMed, Esp@cenet, CA "Phytase, Citrobacter, Phytic acid"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	LEI X.G. & STAHL C.H., 'Biotechnological development of effective phytases for mineral nutrition and environmental protection', In: Appl. Microbiol. Biotechnol., 2001, Vol. 57, pp. 474-481. See the whole document	1 2-9
A	EP 0,779,037 A1 (GIST-BROCADES N.V.) 18 JUNE 1997 See the whole document	1-9
A	US 6,235,517 B1 (FOOD INDUSTRY R. & D. INST.) 22 MAY 2001 See the whole document	1-9
P, X	KIM H.W. et al., 'Isolation and characterization of a phytase with improved properties from Citrobacter braakii', In: Biotechnol. Lett., August 2003, Vol. 25, pp. 1231-1234. See the whole document	1-9

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 JULY 2004 (13.07.2004)	15 JULY 2004 (15.07.2004)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHO, YOUNG GYUN

Telephone No. 82-42-481-8132

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2004/000680

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [X] filed together with the international application in computer readable form

   [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2004/000680

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0779037 A1	18.06.1997	WO 1991/005053 A1	18.04.1991
		US 5863533 A	26.01.1999
		JP 3105920 B2	06.11.2000
		KR 159782 B1	16.11.1998
US 6235517 B1	22.05.2001	TW 457298 B	01.10.2001